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Published in:
Biomicrofluidics

Link to article, DOI:
[10.1063/1.3098319](https://doi.org/10.1063/1.3098319)

Publication date:
2009

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Laiwattanapaisa, W., Yakovleva, J., Bengtsson, M., Laurell, T., Wiyakrutta, S., Meevootisom, V., Chailapakul, O., & Emnéus, J. (2009). On-chip microfluidic systems for determination of L-glutamate based on enzymatic recycling of substrate. *Biomicrofluidics*, 3(1), 014104. <https://doi.org/10.1063/1.3098319>

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On-chip microfluidic systems for determination of *L*-glutamate based on enzymatic recycling of substrate

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(Received 5 November 2008; accepted 18 February 2009; published online 16 March 2009)

Two microfluidic systems have been developed for specific analysis of *L*-glutamate in food based on substrate recycling fluorescence detection. *L*-glutamate dehydrogenase and a novel enzyme, *D*-phenylglycine aminotransferase, were covalently immobilized on (i) the surface of silicon microchips containing 32 porous flow channels of 235 μm depth and 25 μm width and (ii) polystyrene PorosTM beads with a particle size of 20 μm . The immobilized enzymes recycle *L*-glutamate by oxidation to 2-oxoglutarate followed by the transfer of an amino group from *D*-4-hydroxyphenylglycine to 2-oxoglutarate. The reaction was accompanied by reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH, which was monitored by fluorescence detection ($\epsilon_{\text{ex}}=340\text{ nm}$, $\epsilon_{\text{em}}=460\text{ nm}$). First, the microchip-based system, *L*-glutamate was detected within a range of 3.1–50.0 mM. Second, to be automatically determined, sequential injection analysis (SIA) with the bead-based system was investigated. The bead-based system was evaluated by both flow injection analysis and SIA modes, where good reproducibility for *L*-glutamate calibrations was obtained (relative standard deviation of 3.3% and 6.6%, respectively). In the case of SIA, the beads were introduced and removed from the microchip automatically. The immobilized beads could be stored in a 20% glycerol and 0.5 mM ethylenediaminetetraacetic acid solution maintained at a pH of 7.0 using a phosphate buffer for at least 15 days with 72% of the activity remaining. The bead-based system demonstrated high selectivity, where *L*-glutamate recoveries were between 91% and 108% in the presence of six other *L*-amino acids tested. © 2009 American Institute of Physics.
[DOI: [10.1063/1.3098319](https://doi.org/10.1063/1.3098319)]

I. INTRODUCTION

There has been growing interest in the concept of micro-total-analysis systems^{1,2} or laboratory-on-a-chip systems.^{3,4} Microfluidic systems offer several advantages over conventional systems, including less consumption of reagents and energy, short analysis time, and portability.

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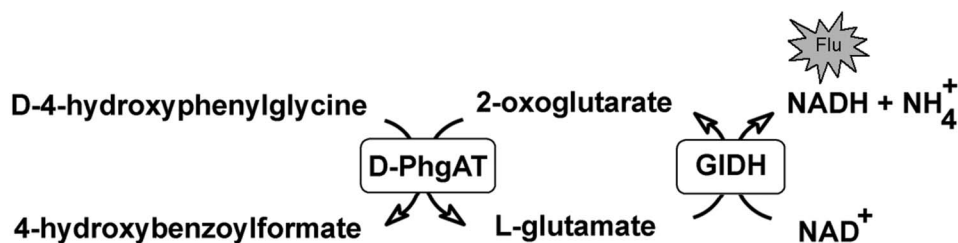


FIG. 1. Enzymatic cycling for the determination of *L*-glutamate using GIDH and *D*-PhgAT. Every turn of the cycle generates one molecule each of NADH and 4-hydroxybenzoylformate. The NADH amplified from the enzymatic reaction (such as that in Fig. 1) is detected by fluorescence ($\epsilon_{\text{ex}}=340$ nm, $\epsilon_{\text{em}}=460$ nm).

Various biological materials, such as DNA and RNA,^{5,6} enzymes,⁷ antibodies,^{8,9} mammalian cells,¹⁰ and micro-organisms,¹¹ can be adapted to these miniaturized systems.

Several applications of microfluidic systems have been documented in the area of chemical analysis for environmental monitoring, medical diagnostic, food, and agricultural industry.

In terms of food safety, *L*-glutamate is one of the most important amino acids to analyze. Chinese restaurant syndrome,¹² Parkinson, and Alzheimer diseases^{13,14} raise the argument of glutamate safety and, therefore, increased attention for determination of *L*-glutamate. Moreover, there is a growing body of literature of *in vivo* *L*-glutamate measurement because it is an important neurotransmitter in the mammalian central nervous system.¹⁵ Therefore, there is a need for sensitive and selective assay for determination of *L*-glutamate in various kinds of samples.

Numerous approaches for analytical determination of *L*-glutamate have been reported in the literature. For example, a common practice is electrochemical monitoring of *L*-glutamate using a combination of enzymes such as *L*-glutamate dehydrogenase (GIDH) with glutamate oxidase.¹⁶ Although selective detection of *L*-glutamate has been achieved by this technique, it requires a sample pretreatment^{16–18} to remove the interfering compounds such as ascorbic acid. Selectivity of this method can be substantially improved by the application of enzymes with high substrate specificity.

In previous studies, we proposed using spectrophotometry¹⁹ and flow injection analysis²⁰ (FIA) for highly sensitive and specific detection of *L*-glutamate. The enzyme *D*-phenylglycine aminotransferase (*D*-PhgAT) with remarkably high substrate specificity, isolated from *Pseudomonas stutzeri* ST201 by our group,^{21,22} was utilized for development of selective *L*-glutamate assay. In combination with GIDH, *D*-PhgAT is able to recycle *L*-glutamate, which results in enhanced assay sensitivity.

The assay systems employed in the present study were based on the enzymatic cycling reactions as shown in Fig. 1. In this system, *L*-glutamate in the sample is converted to 2-oxoglutarate by GIDH with concomitant reduction of nicotinamide adenine dinucleotide (NAD⁺) to NADH. In the subsequent reaction *D*-PhgAT transfers the amino group from *D*-4-hydroxyphenylglycine, which is present in an excess amount, to 2-oxoglutarate and converts it back to *L*-glutamate, thus completing the GIDH reaction cycle. In each round of the reaction cycle, one molecule of NADH is produced and accumulated as the cycle repeats. Each *L*-glutamate molecule can give rise to many NADH molecules since after being oxidized by GIDH, *L*-glutamate can be regenerated and reacts with GIDH again for many rounds. The longer the cycling reaction was allowed to proceed the higher the amount of NADH is generated which can be far more exceeding the amount of *L*-glutamate present in the system. This signal amplification effect brought about by the enzymatic cycling system can greatly enhance the assay sensitivity. On the contrary, in the single enzyme system, the number of NADH molecules formed in the assay using GIDH alone is limited to the number of *L*-glutamate present since each *L*-glutamate molecule is oxidized and gives rise to NADH only one time. After all *L*-glutamate was consumed in the reaction, the signal reaches a plateau and cannot be further increased despite the extension of the reaction time. By formulating proper amounts of the enzymes and substrates in the system,^{19,20} the cycling reaction quickly

attains a steady state where the GIDH and *D*-PhgAT reactions proceed at the same rate. During this steady state, the concentration of *L*-glutamate in the reaction remains constant and the rate of the cycling reaction (thus the rate of NADH accumulation) is directly proportional to the concentration of *L*-glutamate present in the system. Quantitative determination of *L*-glutamate in the sample can be made by comparing the rate of NADH accumulation at the steady state of the assay reaction with those of the standard calibration reactions containing known amounts of *L*-glutamate.

In this work, we expanded this approach onto the field of miniaturized analysis systems. Two types of microfluidic configurations have been constructed for detection of *L*-glutamate based on substrate recycling by *D*-PhgAT and GIDH (Fig. 1). The enzymes were coimmobilized either on (i) a silicon microchip surface or (ii) polystyrene microbeads. Microchip-based systems based on immobilized enzymes,²³ antibodies,^{8,9} and cells^{10,24,25} were previously reported by our group. Microbead-based reactors were earlier described for immunodiagnostics²⁶ and identification of organic explosives²⁷ and show good reproducibility and robustness. In our work, we have demonstrated fully automatic determination of *L*-glutamate in the microfluidic system with good assay characteristics such as selectivity and stability.

II. EXPERIMENTAL

A. Chemicals and buffers

D-PhgAT was purified from a recombinant *E. coli* strain expressing the cloned gene encoding the enzyme from *Pseudomonas stutzeri* ST201 as previously described.²¹

The following reagents were purchased from Fluka Chemie AG (Buchs, Switzerland): β -NAD⁺, pyridoxal-5'-phosphate (PLP), adenosine-5'-diphosphate disodium salt (ADP), *D*-phenylglycine, *D*-4-hydroxyphenylglycine, 2-oxoglutaric acid, benzoylformic acid, and GIDH (EC 1.4.1.3, from bovine liver contained in 50% glycerol). *L*-glutamic acid, glycine, glycerol, ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (Tris), 3-aminopropyltriethoxysilane (APTS, minimum of 98%), polyethylenimine (PEI, 50% w/v aqueous, molecular weight of 750 000), glutaraldehyde (GA, 25% v/v aqueous solution grade I), sodium cyanoborohydride (NaBH₃CN), Bradford reagent, bovine serum albumin (BSA), and *L*-amino acids used in the interference studies were from Sigma (St. Louis, MO). Polystyrene/divinylbenzene Poros beads with active groups (particle size of 20 μ m) were the product of PerSeptive Biosystems (Framingham, MA).

All other chemicals (acetone, sodium hydroxide, hydrochloric acid, ammonia, hydrogen peroxide, and toluene) were of analytical grade. High performance liquid chromatography grade water was prepared in a Milli-Q system (Millipore, Bedford, MA).

The borate buffer (BB) containing 0.01M sodium tetraborate (adjusted to pH 7.0 with 1M HCl) was used for immobilization of enzymes on the surface of silicon microchips. In the case of polystyrene beads, 0.1M potassium phosphate buffer of pH 7.0 was used. The buffer used in the microfluidic systems and for the dilution of standard and sample solutions was 0.1M Tris-HCl, pH 8.0. When not in use, the enzyme microchips were stored at 4 °C in phosphate buffer pH 7.0 in the presence of 20% glycerol and 0.5 mM EDTA.

B. Microfluidic chips

Two types of microfluidic chips were used in the experiment (i) containing 32 porous wall flow channels for immobilization of enzymes and (ii) containing a single flow channel for entrapment of microbeads. The silicon microchips were fabricated by chemical wet etching of silicon, as described previously.^{28,29} The overall dimensions of the first microchip were 13.1 \times 3.1 mm², where two basins (inlet and outlet) with 32 parallel U-shaped channels (length of 10 mm, depth of 235 μ m, width of 25 μ m) were fabricated. The microfluidic channels of the microchip were open, and the chip was thus first covered with the Perspex lid (a sheet of latex film), placed in a Plexiglass holder and then incorporated into the flow system [Fig. 2(a), top].

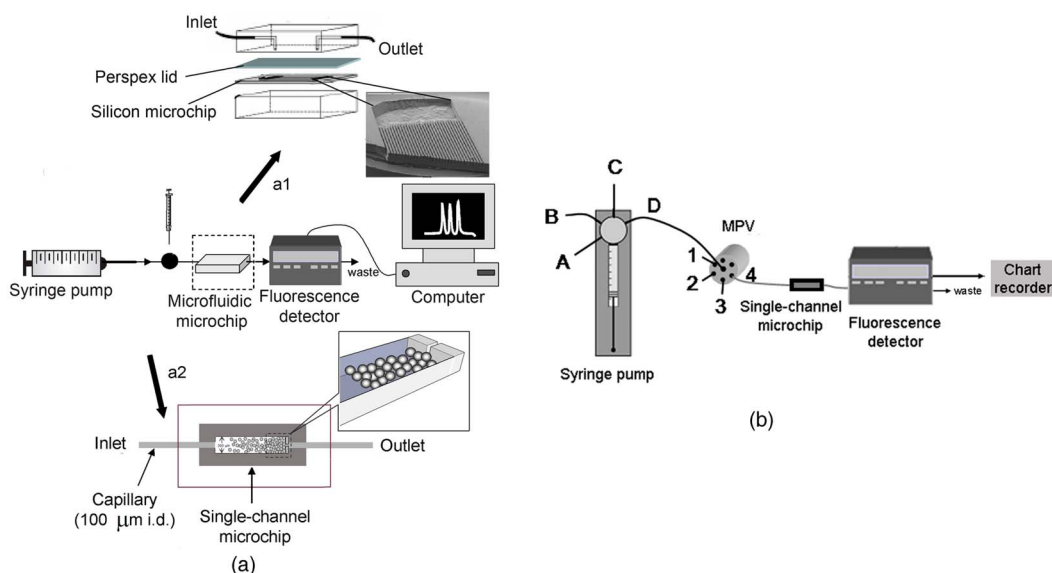


FIG. 2. (a) Schematic of the microfluidic FIA system. A syringe pump was used for carrying the reaction mixture (0.1M Tris-HCl, pH 8.0, 10 mM *D*-4-hydroxyphenylglycine, 1 μ M PLP, and 2 mM NAD⁺). Sample injection was made through a six-port injection valve. The fluorescence detector was connected via the outlet of the microfluidic microchip and the fluorescence peak height signals were recorded using the computer software. a1 shows a detailed view of the flow-through silicon microchip used for coimmobilization of enzymes. a2 shows the single-channel microchip filled with immobilized beads. (b) Configuration of the SIA system. Tube A connects to the syringe pump used for 0.1M Tris-HCl (pH 8.0), tube B is for waste, and tube C is for dispensing and aspirating the samples and reaction mixture to the MPVs. Position 1 on the MPV was occupied by the immobilized bead, 2 by *L*-glutamate sample, 3 by waste, and 4 by connection to the microchip. The fluorescence detector was integrated to monitor the analytical signal followed by the chart recorder to display the fluorescence peak height.

The second type of microchips had overall dimensions of 15×15 mm² and contained a single flow-through channel. The channel is fabricated in 110-silicon anodically etched in KOH, yielding vertical sidewalls (length of 7 mm, width of 300 μ m, depth of 100 μ m). Before the outlet, a grid of standing parallel walls (grid opening of 20 μ m) was microfabricated in the channel. The grid served as a frit to retain enzyme activated beads. The open microfluidic channel was sealed by anodic bonding with a glass lid (borofloat, Schott) to form a robust and nonleaky microfluidic platform. Subsequently capillaries (100 μ m inner diameter) were glued into the inlet and outlet for fluidic interfacing. The above single-channel microchip was integrated with the system by connecting the inlet capillary to the injection valve and the outlet capillary to the fluorescence detector [Fig. 2(b), bottom].

C. Immobilization of enzymes via PEI on silicon surface

The microchips were cleaned before the immobilization of enzymes by the following solutions: (i) A mixture of ammonia (25%), H₂O₂ (30%), and water (1:1:5 by volume) boiled for 5 min and (ii) a mixture of hydrochloric acid (37%), H₂O₂ (30%), and water (1:1:5 by volume) heated to 100 °C for 5 min. The microchips were rinsed thoroughly with water and used immediately for further surface modifications. The cleaned microchips were immersed in 0.5% v/v solution of PEI in BB, stirred at room temperature (RT), and incubated overnight. The chips were subsequently rinsed with BB and the amino groups of the adsorbed PEI were activated in 2.5% v/v GA in BB for 2 h at RT while stirring. The activated microchips were extensively rinsed with Milli-Q and BB to remove traces of GA. For immobilization, the microchip was placed in an enzyme mixture (6 u GIDH and 6 u *D*-PhgAT, 3 mM ADP, 0.5 mM PLP) in BB, and the reaction was allowed to proceed overnight at 4 °C. The Schiff's bases on the microchip surface were reduced by the

addition of NaBH_3CN to the final concentration of 3.5 mg ml^{-1} and the reaction was allowed to proceed for 1.5 h. The immobilized microchip was finally washed thoroughly with Milli-Q and BB.

D. Immobilization of enzymes via APTS on silicon surface

The precleaned microchips, as described in Sec. II C, were silanized in 10% APTS in sodium-dried toluene.³⁰ A vacuum was applied for a few seconds to remove air trapped in the microchip pores, and the reaction was allowed to continue stirring slowly for 1 h at RT in a sealed vessel. The microchip was subsequently removed from the silanization solution and was rinsed several times with toluene, acetone, and BB. All the following steps, including GA activation, enzyme immobilization, residual aldehyde blocking, and Schiff's base reduction, were carried out as described in Sec. II C.

E. Immobilization of enzymes using polystyrene beads

A portion of Poros polystyrene beads with active amino groups (50 mg dry weight) was used for enzyme immobilization. First, the beads were washed twice with 0.1M phosphate buffer, pH 7.0, and then activated by GA 2.5% (v/v) in 1 ml of the same buffer for 3 h at RT. The following steps including enzyme immobilization, residual aldehyde blocking, and Schiff's base reduction were performed as described above. The immobilized beads were suspended in 1 ml of 0.1M phosphate buffer, pH 7.0, and stored at 4 °C until used.

F. Microfluidic FIA system setup and assay procedures

Microchips with immobilized enzymes and beads were incorporated in the flow system as shown in Fig. 2(a), middle. A CMA Microdialysis 100 syringe pump (CMA/Microdialysis, Solna, Sweden) was used to deliver the reaction mixture composed of 0.1M Tris-HCl, pH 8.0, 10 mM *D*-4-hydroxyphenylglycine, 2 mM NAD^+ , and 1 μM PLP at either 20 or 40 $\mu\text{l min}^{-1}$. A Rheodyne six-port valve (Berkeley, CA) equipped with a 1 or 5 μl loop was used for sample injection. The microchip with immobilized enzymes and open porous wall channels was first placed in a specially designed flow cell unit made of Plexiglas with the inlet and outlet tubings glued at both ends. All connections were made using polyetheretherketone (PEEK) tubing (inner diameter of 0.25 mm, Alltech, Deerfield, IL) and a loop volume of 5 μl was used. Introduction of the beads into the microfluidic channels was performed manually using a 1 ml syringe and approximately 100 μl of beads suspension was loaded. The beads were then moved to the microfabricated grid frit region and retained within the channel under visual control. After the measurement, once a decrease in the assay signal was observed, the microchip was disconnected from the system and then the beads were removed from the microchip by introducing a buffer in a reverse direction. Thus, the microchip could be used repeatedly by reloading it with a fresh group of beads. A sample loop volume of 1 μl was used for this system.

L-glutamate standard solutions at concentrations between 0 and 100 μM were introduced into the FIA system via the injection port. In the case of the microchip with immobilized enzymes [Fig. 2(a), top], a carrier flow rate of 20 $\mu\text{l min}^{-1}$ was used. When the *L*-glutamate standard reached the microchip surface, flow was stopped for 5 min, allowing the incubation of the analyte with enzymes. In the following step, the flow rate was increased to 40 $\mu\text{l min}^{-1}$ and a NADH peak was recorded by the fluorescence detector (Hitachi Instruments Inc., model L-7480). Peak height was used to quantify the analyte concentration in the sample. For the microbead reactor [Fig. 2(a), bottom], a flow rate of 20 $\mu\text{l min}^{-1}$ was used throughout all experiments. Data acquisition was performed with PICO software (Pico Technology Limited, Sweden).

G. SIA system setup and assay procedures

For fully automated determination of *L*-glutamate in the microfluidic system, the SIA setup was constructed with an integrated microbead reactor. The system was very promising for auto-

matic injection and removal of the beads from the microchannel. The SIA system presented in Fig. 2(b) consisted of an SG 300 syringe pump (Kloehn Ltd., Las Vegas, NV) equipped with a six-way distribution valve, a CS-1340 EMT ten-port multiposition valve (MPV) (Valco Instruments Co. Inc., Houston, TX), an enzyme microchip, and a fluorescence detector (Hitachi Instruments Inc., model L-7480). The inlet capillary of the single-channel microchip was connected to a ten-position valve. In this system, only four ports of the available ten ports on the MPV valve were used (only beads, sample, waste, and microchip position). The different components of the SIA system were connected by PEEK tubing (0.25 mm inner diameter). The whole system was controlled by a computer using in-house developed software. The fluorescence of NADH peak was recorded by a chart recorder.

The microbead reactor was packed by aspirating and propelling 20 μl of the bead suspension into the microchannel. This step was repeated several times until the channel was completely filled with beads, which can be visually observed. The assay cycle was performed by (i) aspirating 240 μl of the reaction mixture containing the substrates as described previously using the syringe pump, (ii) aspirating 1 μl of *L*-glutamate sample via the MPV into the tubing that connected the MPV and syringe pump, (iii) dispensing of the aspirated sample and the reaction mixture to the microchip with the flow rate of 20 $\mu\text{l min}^{-1}$, and (iv) registration of the corresponding fluorescence signal, which was recorded by a chart recorder. Measurements were performed in triplicate for each sample.

To demonstrate the recycling efficiency of *L*-glutamate, the flow was stopped when the sample reached the microbead reactor to allow amplification of the signal. As microbeads periodically displayed a decrease in enzyme activity with time, they were removed from the microchannel and replaced with a fresh portion.

H. Enzyme activity assay

D-PhgAT activity was assayed as previously described.²⁰ Briefly, 20 μl of *D*-PhgAT was added to 980 μl of a mixture containing 0.1M Tris-HCl, pH 8.0, 1.0 mM *D*-phenylglycine, 2.5 mM 2-oxoglutarate, 5 μM PLP, and 5 μM EDTA. The rate of benzoylformate formation upon transamination of *D*-phenylglycine with 2-oxoglutarate as an amino group acceptor was monitored spectrophotometrically at 254 nm at 25 °C. 1 u of *D*-PhgAT activity was defined as the amount of enzyme capable of producing one micromole of benzoylformate per minute under these conditions.

The activity of GIDH was measured based on the degradation of ammonia and concomitant oxidation of NADH. The depletion of NADH was monitored spectrophotometrically at 340 nm. One activity unit for GIDH corresponded to the amount of enzyme that catalyzed the oxidation of one micromole of NADH per minute under the defined conditions.

I. Protein assay

The protein concentration in the supernatant before and after immobilization was determined by the Bradford method³¹ using BSA as a standard. The resulting enzyme concentrations were used to calculate the coupling yield as the ratio of total protein amount (*D*-PhgAT and GIDH) before and after immobilization.

III. RESULTS AND DISCUSSIONS

A. The choice for carrier support, immobilization method, and assay procedure in the FIA system

Miniaturized flow-through enzyme reactors as described in this work exploits silicon microchips as carrier supports. Due to its mechanical properties, silicon is one of the most widely used materials for microfabrication. However, it is also known for its strong nonspecific adsorption of proteins, which decreases the activity of immobilized molecules.³² For this reason, the surface of a silicon carrier should be modified prior to immobilization of enzymes. Earlier, we have reported immobilization of antibodies on silicon microchips coated with a hydrophilic layer of PEI.⁸ In this

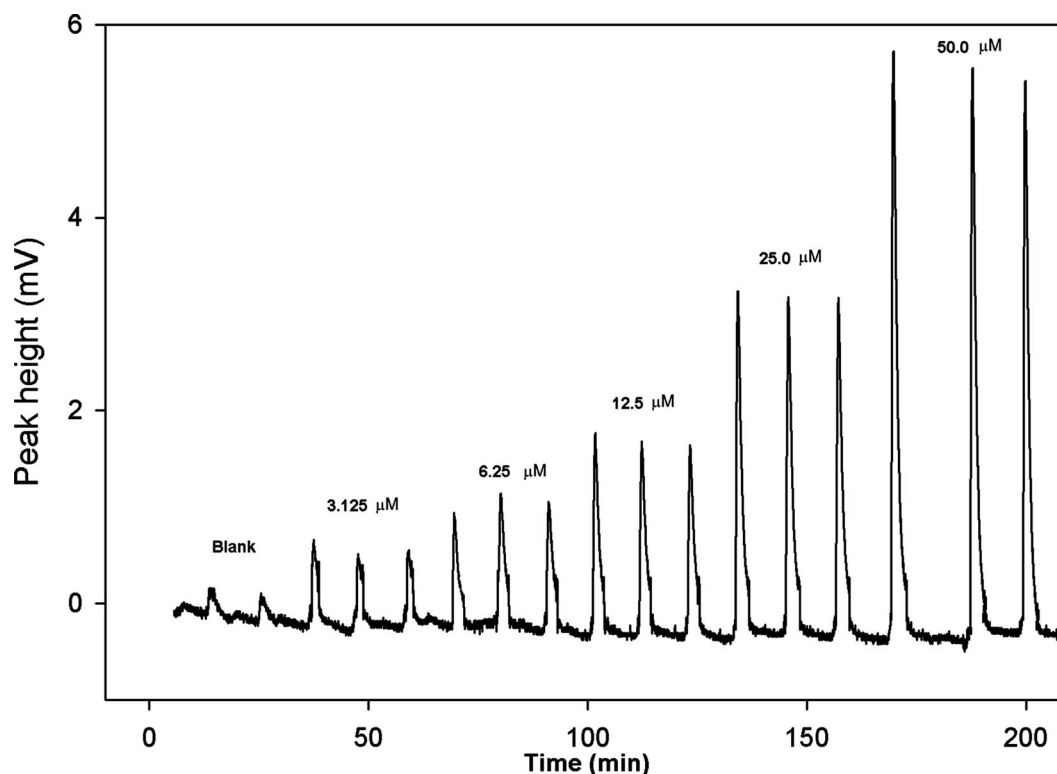


FIG. 3. Typical fluorescence signals for *L*-glutamate standards using the silicon flow-through microchip immobilized enzyme. Each sample was assayed in triplicate and fluorescence peak height from NADH was shown.

work, we applied the same approach for coimmobilization of GIDH and *D*-PhgAT and construction of FIA flow through a microreactor. Due to strong electrostatic interaction between negatively charged silicon surfaces and positively charged PEI chains, the latter adsorbs on silicon in a practically irreversible way.³³ As a reference method,³⁰ the silica surface was derivatized by a layer of APTS. The enzymes were then covalently attached to amino groups of PEI or APTS via GA. Following this approach, the coupling yield for GIDH and *D*-PhgAT was estimated to be 0.8 mg on the microchip. Figure 1 shows recycling of *L*-glutamate by GIDH and *D*-PhgAT with concomitant amplification of NADH, which was detected downstream by fluorescence. Recycling the substrate offers increased assay sensitivity. To maximize substrate recycling and thus the sensitivity, the flow was stopped and injected samples were incubated on the surface of the enzyme microchip for a fixed amount of time. As expected, the incubation time in the microchip influenced the obtained intensity fluorescence signal (data not shown). An incubation time of 5 min was found to be optimal in terms of intensity of the derived signal and assay time for one sample and was thus selected for the subsequent experiments. A typical *L*-glutamate calibration curve following this protocol is shown in Fig. 3. The calibration has a linear range of 3.1–50 μM of *L*-glutamate with a minimum detection limit of 3 μM (calculated using threefold signal-to-noise ratio).

B. Stability of the enzymes on silicon microchips

The immobilized enzymes lost their activity with time. The decay rate is commonly influenced by the immobilization method and the operating conditions for the enzyme reactor. During this study, we employed two different immobilization techniques based on (i) APTS modification of the silicon surface and (ii) PEI modification. The latter was expected to result in higher stability of immobilized enzymes due to its hydrophilic environment. The APTS based procedure has been widely used and is known as the standard method for immobilization on silica surfaces.³⁰

PEI or APTS modified microchips with coimmobilized GIDH and *D*-PhgAT were integrated into the microfluidic FIA system and their stability was evaluated by measuring *L*-glutamate standards over 3 days. The reproducibility of calibration curves over 3 days had 28% and 40.5% relative standard deviation (RSDs) for PEI and APTS microchips, respectively, which indicates a poor stability of the immobilized enzymes. The operational stability of the enzyme microchips was investigated by repetitive injections (30 times) of 12.5 μM *L*-glutamate during a 1 day period. The decrease in activity after the 20 injections was 43% and 64% for PEI and APTS microchips, respectively (results not shown).

To study long-term stability of the microchips, the fluorescence signals from three different concentrations of *L*-glutamate were measured in triplicate every day for 1 month using the PEI modified microchip. Immobilized enzymes displayed a dramatic decrease in activity with 50% of absolute signal after 6 days and 67% after 24 days (data not shown). Due to instability of enzymes immobilized directly on the surface of the silicon microchips, alternative immobilization methods are being investigated.

C. Single-channel microchip: New approach to microfluidic systems

Since enzymes that were directly immobilized on the surface of the silicon microchip displayed a dramatic decrease in activity, an alternative setup using microbeads in a chip was studied. To overcome the disadvantage of instability, GIDH and *D*-PhgAT were coimmobilized on polystyrene beads and packed into the flow-through reactor comprising one microchannel of 7 mm length and 300 μm width. A microbead-packed reactor seems to be practical since the beads can be introduced into the channel and easily removed when the enzyme activity is decreasing. A new portion of beads can subsequently be packed into the channel and subsequent experiments can be performed without losing the activity of immobilized enzymes.

In our experiments, one batch of immobilized enzymes contained 50 mg of the polystyrene beads, which was sufficient for packing the column more than 30 times (using approximately 1 mg of beads for each column). To demonstrate the reproducibility of the system for introduction and removal of beads from the channel, three different concentrations of *L*-glutamate were measured over six columns. The suspension of immobilized beads was introduced and removed from the channel manually using a 1 ml syringe. Good reproducibility was obtained with a RSD of 3.3%. Thus, the microbead-packed enzyme reactor demonstrated superior stability compared to the immobilized enzyme reactor.

D. Storage stability of beads with immobilized enzymes

The immobilized beads were suspended in 0.1M phosphate buffer (pH 7.0) in the presence of 20% glycerol and 0.5 mM EDTA and stored at 4 $^{\circ}\text{C}$ until use. Fluorescent signals from different *L*-glutamate concentrations were regularly measured over 15 days to estimate the storage stability of the beads (Fig. 4). As seen from Fig. 5, the signal decreased rapidly during the first 2 days, reaching $\sim 70\%$. Following this, the signal remained stable during the entire period of measurement. We previously observed similar behavior for a “macroscale” bead-packed reactor.²⁰

E. Integrating the single-channel microchip into the SIA system

As mentioned previously, we demonstrated excellent reproducibility for packing beads into the microfluidic reactor and long-term storage in the manually controlled FIA system. To automate *L*-glutamate assay on the microfluidic chip, the SIA system was configured. The SIA is a recent flow technique for sample and reagent exploitation under computer control, which leads to full automation of the system. Robustness, versatility, simplicity, and low sample and reagent consumption are major advantages of SIA.³⁴ In our work, the introduction and removal of beads, as well as injections of *L*-glutamate and the assay buffer, were all driven in SIA mode as shown in Fig. 2(b). Signal reproducibility after consecutive injections of different *L*-glutamate concentrations was measured in the same way as for the FIA system above. As seen from Fig. 5, good reproducibility of the SIA system using five concentrations of *L*-glutamate with four independent

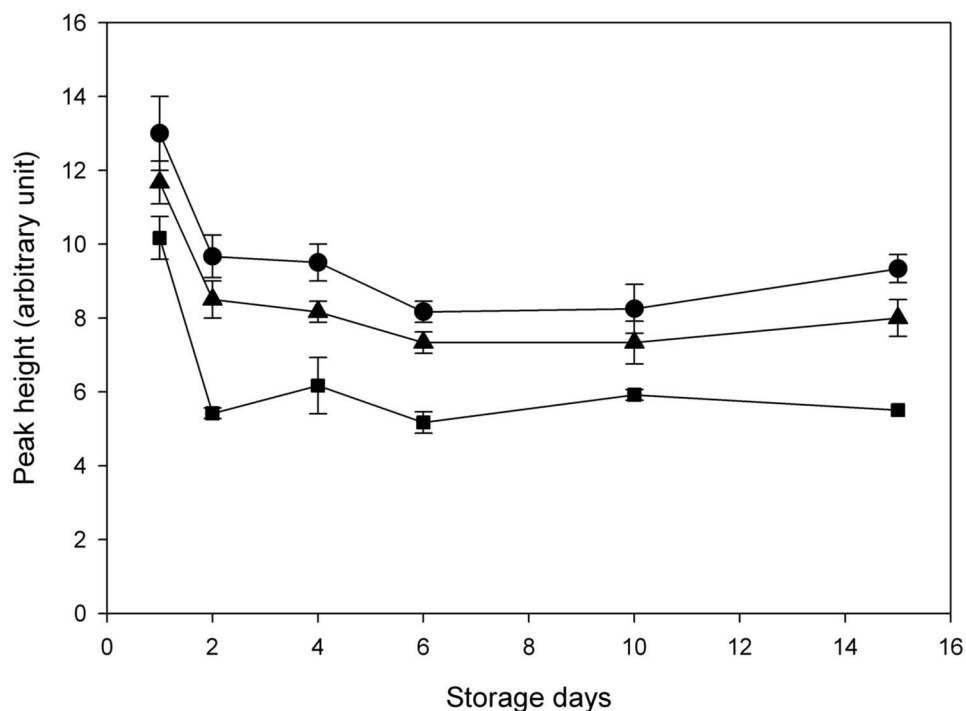


FIG. 4. Storage stability of the immobilized polystyrene beads. Fluorescence signals were obtained from various concentrations of *L*-glutamate at (■) 50, (▲) 75, and (●) 100 μM . Conditions: Operation with microfluidic FIA system integrated with a single-channel microchip and the incubation time of 0 min. Each point is the mean value from triplicate assays; the error bar represents the standard deviation.

enzyme microreactors was obtained with the RSD of 6.6%. Thus, the automatic SIA system was shown to be promising for the routine use of *L*-glutamate analyses. The average limit of detection for *L*-glutamate was calculated to be 3 μM (signal-to-noise ratio of 3).

F. Cycling efficiency of the system

In the present experiments, the SIA system was set up and integrated with the immobilized enzymes in a single-channel microchip. With the microfluidic system, the amount of enzymes in each reactor was generally low. In order to enhance the analytical signal, flow was stopped to allow the analyte, *L*-glutamate, to reside in the reactor for the cycling reaction to proceed for a longer period of time and thus generating more reaction product NADH. For comparison the assay using a *noncycling* system was performed by omitting *D*-4-hydroxyphenylglycine from the reaction mixture while other components were identical to those in the cycling system. Figure 6(a) shows the effect of varying the analyte residence time within the reactor on the intensity of the fluorescence signals comparing from the assay between the cycling and noncycling systems. An incubation time of 3 min was found to be optimal in terms of intensity of the product signal and the assay time for one sample, and this was adopted for the later experiments. *L*-glutamate calibration plots [Fig. 6(b)] illustrate linear proportional response for *L*-glutamate in the 50–200 μM range. The graphs obtained from the cycling and noncycling systems have the slopes of 0.213 and 0.038 peak height unit/ μM *L*-glutamate, respectively. This indicates that at *L*-glutamate concentrations between 50 and 200 μM , the cycling system could amplify the assay signals up to 5.6 times higher than those from the noncycling system.

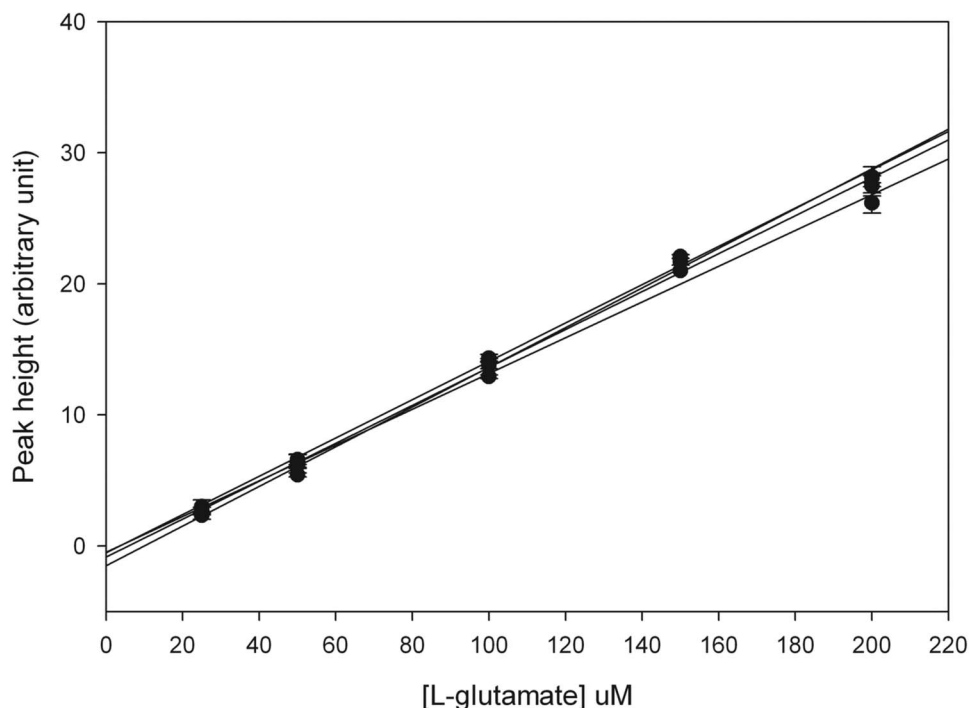


FIG. 5. Reproducibility of the microfluidic SIA system over four *L*-glutamate calibration curves. Good reproducibility was obtained with RSDs of 6.6%. Each point is the mean value from triplicate assays; the error bar represents the standard deviation.

G. Interferences from other amino acids

Generally, a major factor that impairs the specificity of an enzyme based assay is the broad substrate specificity of the enzymes used in the system. Previously, we reported high specificity of *L*-glutamate determination using batch and flow methods based on GIDH and *D*-PhgAT (Refs. 19 and 20) due to the unique property of *D*-PhgAT. This enzyme does not interact with other *L*-amino acids apart from *L*-glutamate. Additionally, it demonstrates high specificity toward *D*-phenylglycine or *D*-4-hydroxyphenylglycine as amino group donors²² both of which are non-natural amino acids not normally found in food. For the microfluidic system, results revealed that the presence of other *L*-amino acids tested including glutamine, aspartate, alanine, phenylalanine, histidine, and tryptophan did not interfere significantly with the recoveries of *L*-glutamate, which were found between 91% and 108%. These values are similar to those obtained for a macroscale bead-packed enzyme reactor, where *L*-glutamate recovery in the presence of other amino acids was within 95%–103%.²⁰ In particular, the system also displayed negligible interferences from ascorbic acid, in which 98% *L*-glutamate recovery was obtained.²⁰ In contrast to other microfluidic systems for *L*-glutamate assay,^{16,18} sample pretreatment to remove ascorbic acid is no longer required. Therefore, our proposed method was advantageous compared to other microfluidic systems for *L*-glutamate assays in terms of simplicity, selectivity, and robustness against amino acid interference commonly encountered in food and biological specimens.

IV. CONCLUSION

In this work, we continued the development of assay systems for determination of *L*-glutamate, addressing the field of microfluidics. FIA systems were developed using GIDH and *D*-PhgAT coimmobilized on (i) silicon microchips with a highly porous surface and (ii) polystyrene beads, which were further packed in a single-channel enzyme reactor. Downsizing the analytical system affected the stability of our immobilized molecules. For instance, system (i) dis-

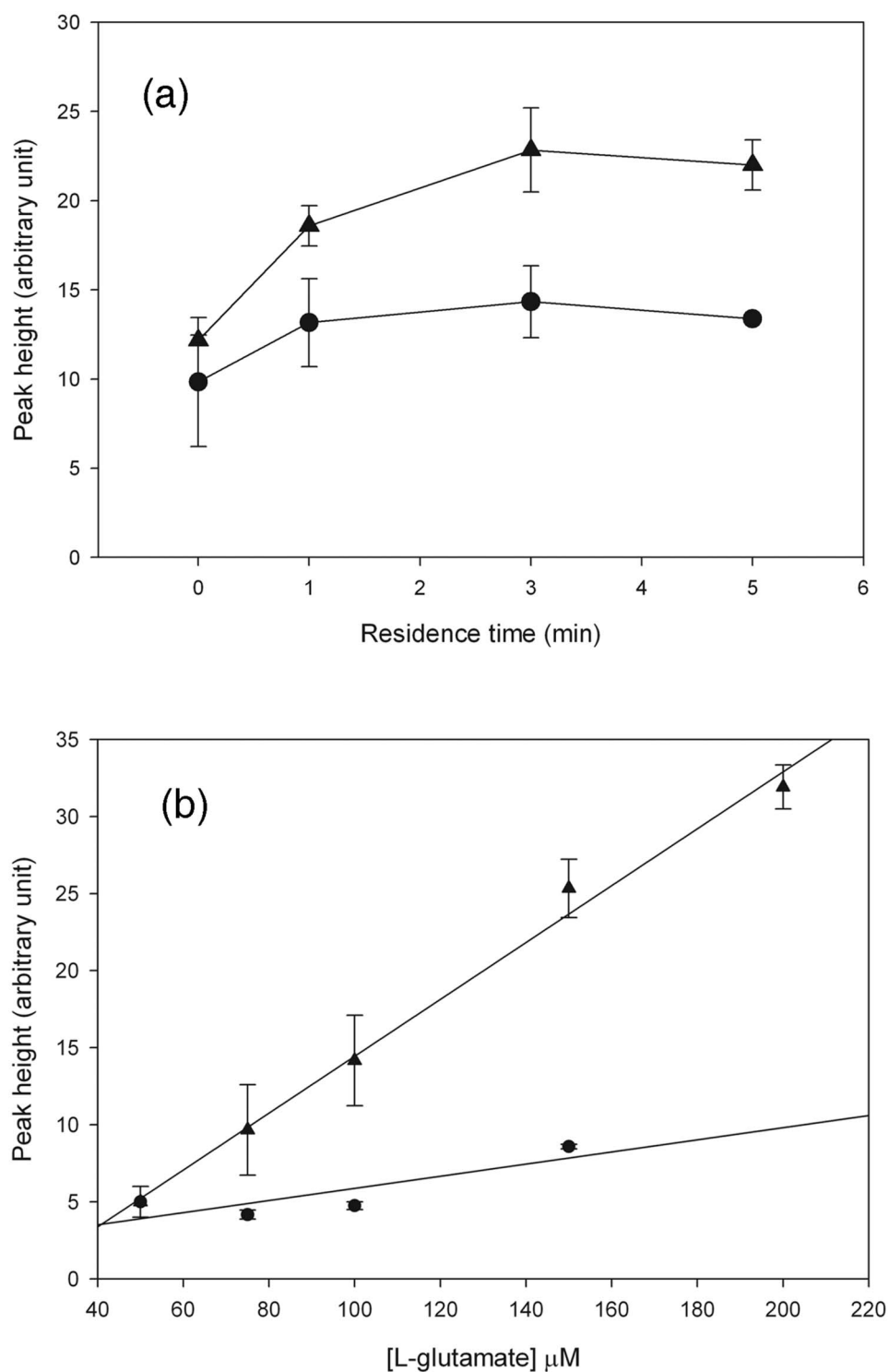


FIG. 6. (a) Effect of the residence time of the *L*-glutamate standard (150 μM) in the enzyme microchip on the intensity of fluorescence signals. (b) Calibration curves for *L*-glutamate using cycling and noncycling microfluidic SIA systems. (▲) Cycling; (●) noncycling system. Each point is the mean value from triplicate assays; the error bar represents the standard deviation.

played a rapid decrease in enzyme activity during short and long-term storage and operations. However, system (ii), consisting of a renewable surface, resulted in acceptable signal stability and is more practical for use. In the latter case, good reproducibility between different bead loadings was obtained (RSD of 3.3%). Both methods resulted in *L*-glutamate quantification at the micromolar level, which is suitable for analysis of food samples. Limit of detection was calculated to be 3 μ M of *L*-glutamate. Recoveries of *L*-glutamate in the presence of several *L*-amino acids were between 91% and 108%, which is practically the same value as for macroscale FIA systems. Therefore, one can conclude that miniaturization of the assay method did not compromise its specificity. The proposed SIA system for assaying *L*-glutamate offers several advantages such as less utilization of enzymes and substrates and excellent reproducibility between runs (RSDs of 6.6%). The SIA implemented in this study is useful for further application by fully automating *L*-glutamate determination in microfluidic systems.

ACKNOWLEDGMENTS

This work was supported by The Swedish Foundation for International Cooperation in Research and Higher Education (STINT), The Swedish Research Council (Vetenskapsrådet), and The Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. program.

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